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RESEARCH

Pelvic pain correlates with peritoneal macrophage abundance not endometriosis

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Abstract

Endometriosis is a chronic neuroinflammatory pain condition affecting ~180 million women worldwide. Surgical removal or hormonal suppression of endometriosis lesions only relieves pain symptoms in some women and symptomatic relapse following treatment is common. Identifying factors that contribute to pain is key to developing new therapies. We collected peritoneal fluid samples and clinical data from a cohort of women receiving diagnostic laparoscopy for suspected endometriosis (n = 52). Peritoneal fluid immune cells were analysed by flow cytometry and data compared with pain scores determined using the pain domain of the Endometriosis Health Profile Questionnaire (EHP-30) in order to investigate the association between peritoneal immune cells and pain symptoms. Pain scores were not different between women with or without endometriosis, nor did they differ according to disease stage; consistent with a poor association between disease presentation and pain symptoms. However, linear regression and correlation analysis demonstrated that peritoneal macrophage abundance correlated with the severity of pelvic pain. $CD14^{high}$ peritoneal macrophages negatively correlated with pain scores whereas $CD14^{low}$ peritoneal macrophages were positively correlated, independent of diagnostic outcome at laparoscopy. Stratification by pain subtype, rather than endometriosis diagnosis, resulted in the most robust correlation between pain and macrophage adundance. Pain score strongly correlated with $CD14^{high}$ (P = 0.007) and $CD14^{low}$ (P = 0.008) macrophages in patients with non-menstrual pain and also in patients who reported dysmennorhea ($CD14^{high}$ P = 0.021, $CD14^{low}$ P = 0.019) or dysparunia ($CD14^{high}$ P = 0.027, $CD14^{low}$ P = 0.031). These results provide new insight into the association between peritoneal macrophages and pelvic pain which may aid the identification of future therapeutic targets.

Lay summary

Endometriosis is a common condition where cells similar to those that line the womb are found elsewhere in the body. It is associated with inflammation and pain in the pelvis and affects ~180 million women worldwide. Current treatments are not effective for all patients and we, therefore, need to understand what causes pain in order to develop new treatments. We investigated the types of immune cells present within the pelvis of women undergoing investigation for suspected endometriosis. Disease diagnosis and stage (I–IV) was recorded along with pain score determined by questionnaire. We characterised the immune cells present and compared them to disease stage and pain score. We found that pelvic pain was linked to the abundance of immune cells but, surprisingly, not to disease stage. These findings suggest that immune cells are closely associated with pain severity in endometriosis and may be good targets for future endometriosis treatments.

Key Words: ▶ endometriosis ▶ macrophage ▶ peritoneal ▶ pain

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Introduction

Endometriosis is a chronic neuroinflammatory condition affecting ~180 million women worldwide and has a socioeconomic impact similar to diabetes (Horne & Saunders 2019). It is defined by the presence of endometrial-like tissue outside the uterus ('lesions'), commonly within the pelvic cavity. The most common symptoms of endometriosis are chronic pelvic pain, dyspareunia, dysmenorrhoea and non-menstrual pain (Horne & Saunders 2019, Zondervan *et al.* 2020). While the exact aetiology of endometriosis is unknown, changes in inflammatory processes are thought to contribute to the pathogenesis of the disease.

The definitive method to diagnose endometriosis is by visualisation at the time of surgery (laparoscopy). Endometriosis is further staged according to the revised scoring system of the American Society for Reproductive Medicine (rASRM) to determine the endometriosis stage based on macroscopic features of the disease including location/appearance of lesions and extent of disease (ranging from I to IV) (1997). Although this staging is commonly used to define disease burden, comparisons between patient-reported severity of pain symptoms and rASRM stage consistently report a poor correlation (Vercellini *et al.* 2007). For example, a multivariate analysis of over 1000 patients assessing the correlation between endometriosis stage, lesion type and pain severity demonstrated that deep endometriosis is associated with pain symptoms characteristic of anatomical location (dyspareunia) but evidence for an association of other pain subtypes (dysmenorrhea, non-menstrual pain) with disease severity/location was marginal (Vercellini *et al.* 2007). Dysmenorrhea is reported to be more frequent and to result in higher pain scores in women with endometriosis but this is also independent of lesion location (Fauconnier & Chapron 2005). Notably, pelvic pain symptoms are common, with up to 90% of women reporting having experienced dysmenorrhea, 42% dyspareunia and 39% acyclic (non-menstrual) pain (Jamieson & Steege 1996) at some time in their lives. The relationship between pain symptoms and diagnosed endometriosis is further complicated by the identification of lesions in asymptomatic women (Fauconnier & Chapron 2005). These data demonstrate that identifying potential pathophysiological mechanisms responsible for pain, beyond those associated with endometriosis lesions, remains important as a step towards improved patient-focused therapies.

Peritoneal macrophages are the most abundant immune cell in the peritoneal cavity (Kubicka *et al.* 1996) with defined roles in clearance of apoptotic cells and immune surveillance. Analysis of peritoneal fluid from women with endometriosis suggests that the inflammatory microenvironment is altered in the peritoneal cavity of women with disease. Notably, increased concentrations of macrophage growth factors and chemokines such as colony-stimulating factor 1 (CSF1) and monocyte chemoattractant protein 1 (CCL2) are detected in peritoneal fluid from women with endometriosis (Arici *et al.* 1997, Budrys *et al.* 2012, Beste *et al.* 2014) and this is associated with increased numbers of peritoneal macrophages compared to women without disease (Hill *et al.* 1988, Beste *et al.* 2014, Gogacz *et al.* 2017). Furthermore, peritoneal macrophage function appears to be dysregulated in endometriosis patients and this is characterised by increased secretion of pro-inflammatory cytokines and reduced phagocytic capacity (Chuang *et al.* 2009, Punnonen *et al.* 1996, Richter *et al.* 2005, Beste *et al.* 2014). Peritoneal macrophage dysfunction is thought to contribute to the establishment of lesions in women with endometriosis, although the mechanistic evidence for this is largely based on studies in mouse models. These studies demonstrate that peritoneal macrophages are increased in experimentally induced endometriosis and are required for the vascularisation and growth of endometriosis lesions (Bacci *et al.* 2009, Capobianco *et al.* 2011).

Macrophage dysfunction may also affect pain symptoms via secretion of neurotrophic factors that promote nerve growth (Kajitani *et al.* 2013). Notably, CD68-positive macrophages are reported to co-localise with nerve fibres within peritoneal endometriotic lesions and it has been speculated that they can promote endometriosis pain symptoms in women (Tran *et al.* 2009). In a mouse model of endometriosis, peritoneal macrophages appeared to promote endometriosis-associated hyperalgesia, putatively via secretion of macrophage-derived insulin-like growth factor 1 (Forster *et al.* 2019).

Macrophages are highly diverse and we need a better understanding of the different subpopulations present in endometriosis in order to determine if they have distinct functional roles. Studies in other contexts have reported there is heterogeneity within human peritoneal macrophage populations and at least two subsets can be identified by assessing the expression of canonical markers, such as CD14, CD16 and HLA-DR (Ruiz-Alcaraz *et al.* 2016, 2018) or complement receptor of the immunoglobulin superfamily (CRIg) (Irvine *et al.* 2016). Notably, these peritoneal macrophage subsets

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appear to be altered in disease states, such as liver cirrhosis (Irvine *et al.* 2016).

The objective of this study was to phenotype and quantify peritoneal macrophage subpopulations in women undergoing diagnostic laparoscopy for suspected endometriosis and to determine whether this was associated with the presence of endometriosis and/or pain symptoms.

Materials and methods

Study approval

Written informed consent was obtained from all study participants prior to surgery; ethical approval was granted by the Lothian Research Ethics Committee (LREC 11/AL/0376). Methods were carried out in accordance with NHS Lothian Tissue Governance guidelines and EPHect guidelines (https://endometriosisfoundation.org/ephect/).

Clinical samples and patient data

Eligible participants were women with chronic pelvic pain (aged 18–50 years) of >3 months duration who were undergoing diagnostic laparoscopy for suspected endometriosis in NHS Lothian, UK. Pelvic pain was defined as pain located within the true pelvis (between and below the anterior iliac crests). Screening commenced on 23/10/2014 and last participant recruited on 03/05/2016. Of the 202 women who were approached for participation, 121 consented to take part. Peritoneal fluid was recovered from 74 women during surgery, of which 52 had sufficient volume for downstream analysis. 22 participants were excluded from the study due to insufficient peritoneal fluid volume. Pain scores were determined using the pain domain of the Endometriosis Health Profile Questionnaire (EHP-30) (a validated endometriosis-specific pain questionnaire completed pre-operatively (20)). Age, BMI, menstrual cycle stage and hormone status were obtained and recorded along with other key clinical data (Supplementary Tables 1 and 2, see section on supplementary materials given at the end of this article). For 29 women who were not on hormones and had regular menstrual cycles, the diagnosis of endometriosis was confirmed macroscopically at laparoscopy in 19 women, whereas no evidence of endometriosis was found in ten women ('no endo'). Women with endometriosis diagnosis were subsequently classified according to the revised scoring system of the American Society for Reproductive Medicine (rASRM) as stage I (n= 10), II (n= 4) and III/IV (n= 5). For some analyses, the remaining 25 women who were receiving hormone treatment were included. In women who met this criterion, diagnosis of endometriosis was confirmed in 14 women, while 11 were classified as 'no endo' (no obvious pelvic pathology at laparoscopy).

Flow cytometry

Immune cells recovered from peritoneal fluid were analysed by flow cytometry using standard methods (McKinnon 2018). Briefly, cells were washed and subjected to red cell lysis before being counted and resuspended in FACS buffer at a concentration of 0.5–1 × 10^6 cells/100 µL. Antibodies were used at manufacturer's recommended concentration (Supplementary Table 3). Cells were gated to select immune cells (live, $CD45^+$) and further sub-gated to select myeloid cells ($CD11b^+$) and exclude T, B and NK cells ($CD3^-$, $CD19^-$, $CD56^-$). Gating of subpopulations was performed based on fluorescence minus one (FMO) and population distribution (CD14). This population gating was applied to all samples. Samples were assessed using a Becton Dickinson FACS Aria II. Data were analysed using FlowJo™ Software.

Statistical analysis

Statistical analysis was performed using Graphpad prism. T test was used to test the difference in the means of two groups. Two-way ANOVA was used to determine the significance between treatments in grouped data. Non-parametric testing was utilised where sample sizes were insufficient to confirm normality of data distribution; Kruskal–Wallis test was used to assess differences between multiple groups or Mann–Whitney test to assess variance between two groups. Pearson correlation was used to quantify the degree to which two variables (macrophages abundance and pain score) were related. Criterion for significance was $P< 0.05$. All data are presented as mean ± S.E.M.

Results

Pelvic pain does not correlate with endometriosis diagnosis or disease stage

Baseline characteristics of the patient cohort were assessed to identify potential factors which may influence pelvic

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pain in women. Pain scores were determined using the pain domain of the EHP-30 questionnaire (Jones *et al.* 2001). For women who were not on hormones; Age, BMI, and pain scores did not differ between those women categorised as 'no endo' or those with surgically diagnosed endometriosis (Fig. 1A and Supplementary Table 1). There was no statistically significant difference in pain score between endometriosis rASRM stages I, II or III/IV, although pain scores were highly variable (Fig. 1B). Oral contraceptives and treatments which suppress hormone production are reported to decrease endometriosis-associated pain in some women (Taylor *et al.* 2017, Crosignani *et al.* 2006). In this study, pain scores in women receiving hormonal treatment (H) were not different from those women who were not on hormones (NH) and this was true in women with (Fig. 1C) and without (Fig. 1D) a confirmed surgical diagnosis of endometriosis.

Two populations of peritoneal macrophages are present in the peritoneal fluid of women with suspected endometriosis which are not altered by hormone status

Cells were recovered from peritoneal fluid and analysed by flow cytometry. Cells were gated to select immune cells and further sub-gated to select myeloid cells and exclude T, B and NK cells (Fig. 2A and B). Two populations of peritoneal macrophages (PMφ) were identified which were HLA-DR-positive and had either high or low-intensity expression of CD14 (Fig. 2C).

In women with regular menstrual cycles, the phase of the menstrual cycle (categorised as proliferative or secretory), had no significant impact on the abundance of CD14^{high} or CD14^{low} PMφ populations (Fig. 2D). Although hormone treatment did not alter the abundance of any of these immune cell populations (Fig. 2E and Supplementary Fig. 1), samples from patients on hormones were excluded from the subsequent analysis to avoid any confounding effects of this variable on other disease parameters.

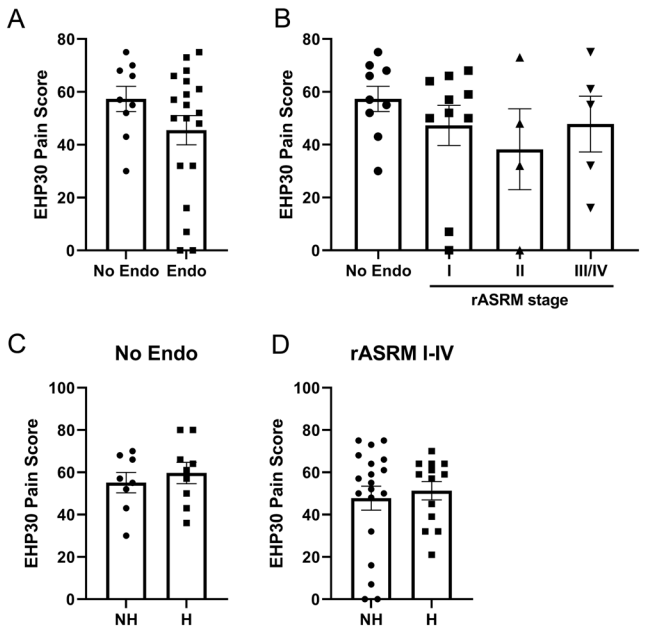

Figure 1 Pain score does not differ by endometriosis diagnosis, stage of disease or hormone treatment. (A) Presence/absence of endometriosis did not affect the pain score (pain domain of EHP30) and (B) did not differ according to rASRM stage. Hormone treatment did affect pain score in women without (C) or with endometriosis diagnosis (D).

Peritoneal macrophages are not altered by infertility or heavy menstrual bleeding

We next assessed whether the co-morbidities infertility or heavy menstrual bleeding (HMB), recorded as part of patient cohort data collection, could affect the abundance of PMφ subpopulations. Infertility did not alter the abundance of CD14^{high} or CD14^{low} macrophages in women with endometriosis (Fig. 2F). PMφ populations were not altered by the presence of HMB (Fig. 2G).

CD14^{high} peritoneal macrophages are increased in women with endometriosis

PMφ were assessed in all women within the cohort who had regular cycles and were not receiving hormone treatment and comparisons made between 'No endo' and those with endometriosis (pooled 'rASRM I–IV'). CD14^{high} PMφ were significantly higher in women with endometriosis (rASRM I–IV) compared to women without disease (Fig. 3A, $P < 0.05$). No significant difference was found for CD14^{low} PMφ, although the average appeared lower for women with endometriosis (Fig. 3B). To assess the relationship between the two populations we calculated the ratio of CD14^{high} to CD14^{low} macrophages. CD14^{high} PMφ were the predominant population in the peritoneal cavity and this difference was more pronounced in women with endometriosis (Fig. 3C) and with increasing rASRM stage (Fig. 3D). Analysis according to rASRM stage (Fig. 3C) identified a significant difference between subpopulations of CD14^{high} and CD14^{low} PMφ in women classified as rASRM stage I ($P < 0.01$), II ($P < 0.0001$) or III/IV ($P < 0.001$). No significant difference was found between CD14^{high} and CD14^{low} PMφ in women without endometriosis.

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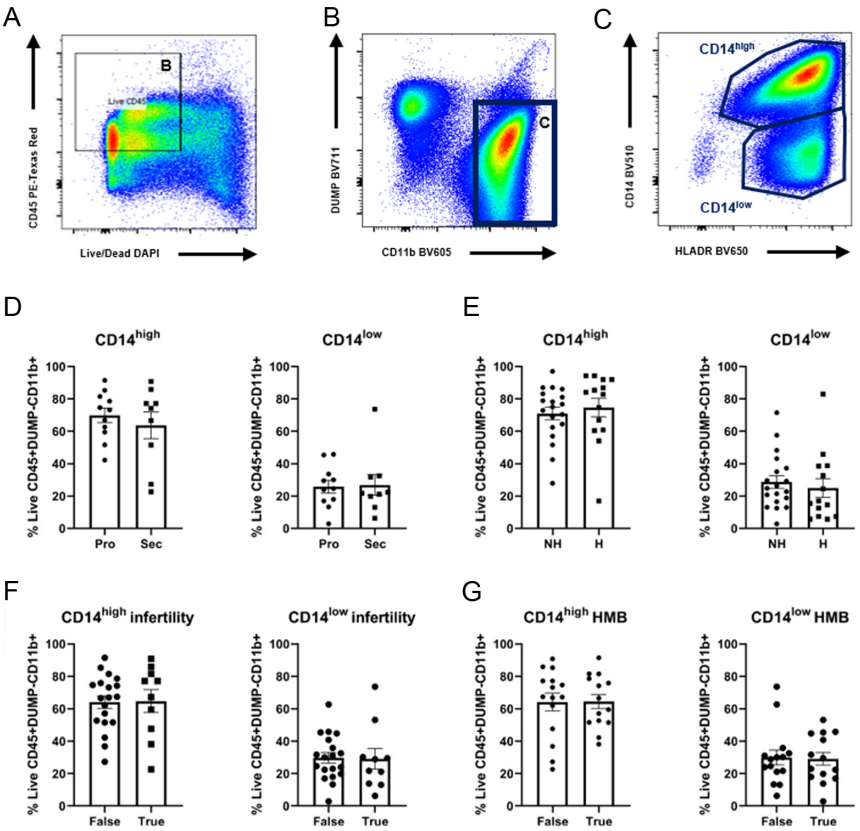

Figure 2 Analysis of peritoneal fluid immune cells from women with diagnosed endometriosis reveals two populations of peritoneal macrophages that are not altered by hormones or other reproductive health disorders. Representative flow cytometry plots from pelvic peritoneal fluid (PF) immune cells. Cells were gated to select; (A) Live, CD45$^+$ (all immune cells) and (B) CD11b$^+$ (myeloid cells), CD3$^-$CD19$^-$CD56$^-$ ('DUMP'; T, B and NK cells). (C) Two peritoneal macrophage (PMφ) subpopulations were identified that were HLA-DR$^+$ and CD14$^+$ (High vs low). (D) Phase of the menstrual cycle (Pro: proliferative; Sec: secretory) did not affect the abundance of CD14^{high} (*t* test, $P = 0.5035$) or CD14^{low} cells (Mann–Whitney test, $P = 0.7664$) thus samples were thereafter not stratified on this basis. (E) The abundance of CD14^{high} (Mann–Whitney test, $P = 0.3723$) or CD14^{low} cells (Mann–Whitney test, $P = 0.3345$) did not differ if women were not on hormones (NH) or on a hormonal treatment (H). (F) The abundance of CD14^{high} or CD14^{low} peritoneal macrophages (PMφ) did not differ between women reporting infertility (True) and those who did not (False). (G) The abundance of CD14^{high} or CD14^{low} PMφ in women with endometriosis did not differ between women reporting HMB (True) and those who did not (False).

Peritoneal macrophages correlate with pelvic pain scores in women with endometriosis and are influenced by pain subtype

The abundance of either CD14^{high} or CD14^{low} PMφ was compared to pain scores in women with endometriosis who were not on hormones and Pearson correlation calculated. In women who were diagnosed with endometriosis (rASRM I–IV), CD14^{high} PMφ did not significantly correlate with pain score (Fig. 4, All, $P = 0.057$), although a trend for negative correlation was evident. However, CD14^{low} PMφ showed a moderate positive relationship with the pain score that was borderline significant (Fig. 4, All, $P = 0.047$). No correlation was observed in women without endometriosis, irrespective of hormone status, but these subgroups had the fewest patients (Supplementary Fig. 2).

To account for different pain subtypes within women with endometriosis, patients were stratified based on reported presence of pain subtypes; dysmenorrhea (DM), dyspareunia (DP) and non-menstrual pain (NMP). Dysmenorrhea was present in the majority of endometriosis patients (89.5%, Supplementary Table 4) and comparisons were therefore similar to those that accounted for all endometriosis patients; CD14^{high} PMφ did not significantly correlate with pain score (Fig. 4, DM, $P = 0.057$) but CD14^{low} PMφ showed a significant positive relationship with pain score (Fig. 4, DM, $P = 0.049$).

Dyspareunia was less common than dysmenorrhea in endometriosis patients (73.7%, Supplementary Table 4). CD14^{high} PMφ had a significant negative correlation with pain score (Fig. 4, DP, $P = 0.044$) and CD14^{low} PMφ had a significant positive correlation with pain score (Fig. 4, DP, $P = 0.031$).

Non-menstrual pain (NMP) was commonly reported in endometriosis patients (84.2%, Supplementary Table 4). CD14^{high} PMφ had a significant negative correlation with pain score (Fig. 4, NMP, $P = 0.015$) and CD14^{low} PMφ had a significant positive correlation with pain score (Fig. 4, NMP, $P = 0.015$).

Based on calculated correlation coefficient values (r), the strongest relationship between PMφ and pain scores was in women with endometriosis who reported experiencing pain outwith menses (non-menstrual pain; NMP). Notably, multiple pain subtypes were present in many patients and stratification was therefore based on the presence of a given subtype. To account for potential interactions between pain subtypes, correlations were also calculated on the basis of whether patients reported both DM and DP, DM and NMP, DP and NMP or all three

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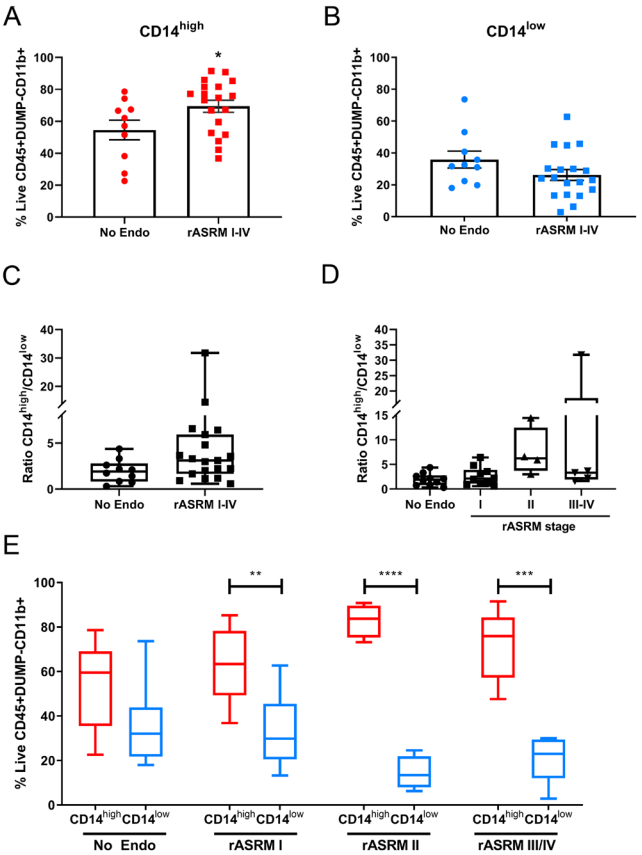

Figure 3 CD14^{high} peritoneal macrophages are increased in women with endometriosis. (A) Frequency of CD14^{high} peritoneal macrophages (PMφ) was greater in women with endometriosis (rASRM stages I–IV, $n = 19$, t test, $P < 0.05$) than those without endometriosis ('No endo'; $n = 10$). (B) Frequency of CD14^{low} PMφ was not significantly different in women with endometriosis compared to those without disease. (C) The ratio of CD14^{high} to CD14^{low} PMφ was greater in women with endometriosis (rASRM I–IV) than those without endometriosis (no endo). (D) The ratio of CD14^{high} to CD14^{low} PMφ was greater in women from each rASRM stage than those without endometriosis (no endo). (E) CD14^{high} PMφ were significantly higher than CD14^{low} PMφ in women with endometriosis stratified according to rASRM I ($n = 10$, $P < 0.01$), rASRM II ($n = 4$, $P > 0.0001$) and rASRM III/IV ($n = 5$, $P > 0.001$) but not in women without endometriosis ($n = 9$; two-way ANOVA). *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$, ****$P < 0.0001$.

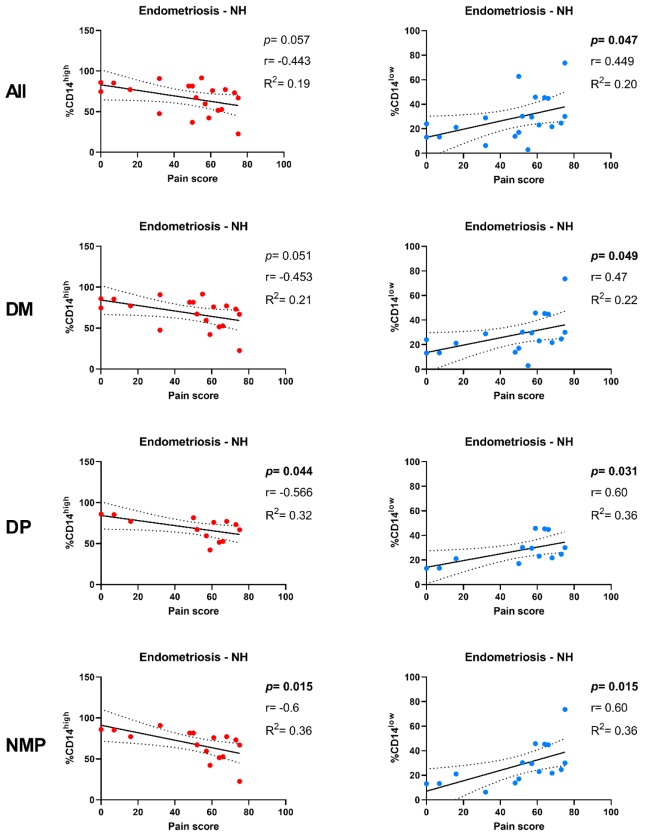

Figure 4 Peritoneal macrophages correlate with the severity of pelvic pain in women with endometriosis and are influenced by pain subtype. Frequency of CD14^{high} PMφ did not significantly correlate with pain scores ($n = 20$, $P = 0.057$) but CD14^{low} PMφ showed a significant positive correlation with pain score in endometriosis patients ($n = 20$, $P = 0.047$). Patients were stratified based on reported presence of pain subtypes; dysmenorrhea (DM), dyspareunia (DP) and non-menstrual pain (NMP). Endometriosis patients who reported dysmenorrhea showed moderate positive correlation between CD14^{low} PMφ and pain score ($n = 19$, $P = 0.049$) but no significant correlation between CD14^{high} PMφ and pain score. Endometriosis patients who reported dyspareunia showed a significant negative correlation between CD14^{high} PMφ and pain score ($n = 13$, $P = 0.0441$) and a significant positive correlation between CD14^{low} PMφ and pain score ($n = 13$, $P = 0.0301$). Endometriosis patients who reported non-menstrual pain showed a significant negative correlation between CD14^{high} PMφ and pain score ($n = 16$, $P = 0.0147$) and a significant positive correlation between CD14^{low} PMφ and pain score ($n = 16$, $P = 0.0146$). P value, correlation coefficient (r) and goodness of fit (R^2) calculated by Pearson correlation. Line of best fit calculated by simple linear regression (black line) with 95% CIs (dashed line). NH, not on hormones. Attribution of pain subtype was not exclusive as multiple subtypes were reported in some patients.

subtypes but this did not affect the correlations reported by individual pain subtypes (Supplementary Table 4).

Peritoneal macrophages correlate with severity of pelvic pain in women irrespective of endometriosis diagnosis

To extend the data on women with endometriosis, the abundance of either CD14^{high} or CD14^{low} PMφ was also compared to pain scores using data from all women in our cohort who were not on hormones (No endo and rASRM I–IV combined) and Pearson correlation calculated (Fig. 5). A significant correlation was found for all patients for both CD14^{high} (Fig. 5, All, $P = 0.031$), and CD14^{low} PMφ (Fig. 5, All, $P = 0.032$) with pain score. This was also true when data were stratified by pain subtype.

Dysmenorrhea was present in most patients (80%, Supplementary Table 4). In women with dysmenorrhea, CD14^{high} PMφ negatively correlated with pain score (Fig. 5, DM, $P = 0.021$) and CD14^{low} PMφ showed a significant

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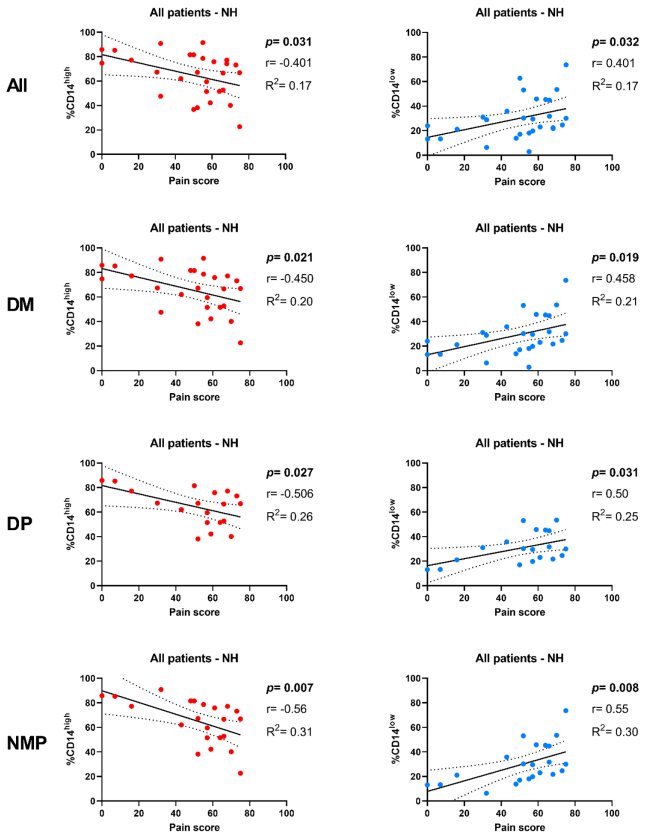

Figure 5 Peritoneal macrophages correlate with the severity of pelvic pain in women with suspected endometriosis irrespective of diagnosis. Frequency of CD14^{high} PMφ significantly negatively correlated with pain score (n = 28, P = 0.031) and CD14^{low} PMφ showed a significant positive correlation with pain score (n = 28, P = 0.032) in all patients irrespective of the endometriosis diagnosis. Patients were stratified based on reported presence of pain subtypes; dysmenorrhea (DM), dyspareunia (DP) and non-menstrual pain (NMP). Patients who reported dysmenorrhea showed a significant negative correlation between CD14^{high} PMφ and pain score (n = 26, P = 0.0212) and a significant positive correlation between CD14^{low} PMφ and pain score (n = 26, P = 0.01865). Patients who reported dyspareunia showed a significant negative correlation between CD14^{high} PMφ and pain score (n = 19, P = 0.0270) and a significant positive correlation between CD14^{low} PMφ and pain score (n = 19, P = 0.0306). Patients who reported pain non-menstrual pain showed a significant negative correlation between CD14^{high} PMφ and pain score (n = 22, P = 0.0074) and a significant positive correlation between CD14^{low} PMφ and pain score (n = 22, P = 0.0083). P value, correlation coefficient (r) and goodness of fit (R^2) calculated by Pearson correlation. Line of best fit calculated by simple linear regression (black line) with 95% confidence intervals (dashed line). NH, not on hormones. Attribution of pain subtype was not exclusive as multiple subtypes were reported in some patients.

positive relationship with pain score (Fig. 5, DM, P=0.019). Dyspareunia was less common than dysmenorrhea (70%, Supplementary Table 4). CD14^{high} PMφ had a significant negative correlation with pain score (Fig. 5, DP, P= 0.027) and CD14^{low} PMφ had a significant positive correlation with pain score (Fig. 5, DP, P= 0.031). Non-menstrual pain (NMP) was commonly reported (76.7%, Supplementary Table 4). CD14^{high} PMφ had a strong significant negative correlation with pain score (Fig. 5, NMP, P= 0.007) and CD14^{low} PMφ had a significant positive correlation with pain score (Fig. 5, NMP, P= 0.008). Similar to the analysis of data from the subgroup of women who were diagnosed with endometriosis, when all patients were considered the strongest relationship between PMφ and pain scores was in women who reported experiencing non-menstrual pain (CD14^{high} r=–0.56; CD14^{low}, r=0.55).

Discussion

Peritoneal macrophages are believed to play a fundamental role in the pathophysiology of endometriosis (Bacci *et al.* 2009, Yuan *et al.* 2016, Horne & Saunders 2019, Zondervan *et al.* 2020) but to date, information regarding different phenotypes of macrophage and how they relate to pain symptoms in women has been limited. In this study, we performed multi-parameter flow cytometry analysis of peritoneal fluid immune cells from women undergoing a diagnostic laparoscopy for suspected endometriosis. We believe our data are the first to demonstrate that a CD14^{high} PMφ subset are increased in women with laparoscopically diagnosed endometriosis and that the abundance of both CD14^{high} and CD14^{low} PMφ populations correlate with pain symptoms independent of the endometriosis diagnosis.

In a previous study, researchers reported an increase in the abundance of macrophages in the peritoneal fluid of 37 women with endometriosis based on the expression of the macrophage marker CD68 (Beste *et al.* 2014) which is consistent with the subset specific increase in CD14^{high} detected in the current study. Based on relative abundance and comparative transcriptional profiling in other studies (Irvine *et al.* 2016), we believe CD14^{high} and CD14^{low} to be broadly equivalent to large (LpM) or small (SpM) peritoneal macrophage populations described in mice (Cassado *et al.* 2015). In response to peritoneal cavity inflammation in mice, LpM decrease and this is associated with infiltration of monocytes that replenish the LpM pool. Notably, it is not possible to distinguish between 'resident' PMφ and infiltrating monocytes in women as they both express CD14. Thus, the increase in CD14^{high} cells we detected in women with endometriosis may in part be accounted for by monocyte infiltration. This may also be reflected by the increase in the proportion of CD14^{high} relative to CD14^{low} we detected with rASRM stages I, II and III/IV which may be associated with increased peritoneal inflammation in response to disease burden. Future studies will need to

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profile paired monocyte and PMφ samples from women with endometriosis in order to distinguish their respective contribution to the altered peritoneal microenvironment that exists in women with endometriosis.

We assessed pain scores in 52 women who were scheduled to have a diagnostic laparoscopy for suspected endometriosis. Pelvic pain symptoms were recorded using the pain dimension of the EHP-30 questionnaire, an extensively validated endometriosis-specific health-related quality of life measurement (Jones *et al.* 2004, Bourdel *et al.* 2019). We found that pain scores in our cohort of women with endometriosis (mean and S.E.M., 45.5 ± 5.54) were comparable to previous data generated using the EHP-30 questionnaire in larger cohorts of women from the UK (mean pain score 43.3, n= 594 women), USA (mean pain score 49.3, n= 225 women) and Australia (mean pain score 40.7, n= 189 women) (Jones *et al.* 2006, Jenkinson *et al.* 2008, Khong *et al.* 2010) so we believe they are representative of this patient group. These previous studies focused on pain scores in women with surgically diagnosed endometriosis but in the current study, we also analysed pain scores in women who did not receive a diagnosis of endometriosis at laparoscopy. Interestingly, mean pain score in the 'no endo' group was higher (mean and S.E.M., 57.3 ± 4.78) than women with endometriosis but variation within groups meant this was not statistically significant when compared to pain scores in women with endometriosis. Pain scores were also not significantly different between patients graded as rASRM stage I (47.3), II (38.3) or III/IV (47.8). Thus, in the current patient cohort, neither a diagnosis of endometriosis nor rASRM stage affected pain scores. Taken together, these data confirm previous findings that pain symptoms do not correlate well with disease presentation (Vercellini *et al.* 2007).

We profiled peritoneal cavity immune cells to determine if changes in the inflammatory environment could predict the severity of pain symptoms. Broadly, we found a negative correlation between $CD14^{high}$ PMφ and pelvic pain and a positive correlation between $CD14^{low}$ PMφ and pelvic pain. These correlations were significant in patients with endometriosis but also when the whole patient cohort was evaluated independently of the endometriosis diagnosis. The correlation between the abundance of peritoneal macrophage subsets and pain score was more pronounced when patients were stratified by pain subtype. Patients who reported non-menstrual pain showed a marked significant correlation between peritoneal macrophages and pain score and these patients also showed the strongest correlation coefficient. This suggests a potential disconnect between pain and menstrual dysfunction, reinforcing the possibility that inflammatory changes in the peritoneal environment may be a greater driver of pain symptoms in some patients. Thus, although some patients may have no 'active' or visible endometriosis at the time of laparoscopy they may exhibit detectable changes in macrophages which correlate to pelvic pain.

A previous study reported a correlation between concentrations of IL6 in peritoneal fluid and pelvic pain symptoms in women with endometriosis (Velasco *et al.* 2010). Notably, PMφ are reported to be the principle source of IL-6 and this cytokine is increased in peritoneal fluid in women with endometriosis (Wu *et al.* 1999). Pain symptoms may therefore be affected by secretion of pro-inflammatory cytokines from PMφ in response to 'active' disease or as a legacy of previous inflammation from 'historic' disease. Thus, further profiling of peritoneal macrophages to account for both their phenotype and function could provide new insights into the relationship between endometriosis and pelvic pain symptoms. Notably, accounting for pain subtype as well as the severity of pelvic pain symptoms will provide the greatest resolution for identifying specific mediators with the potential to promote inflammatory pain.

Current treatment approaches for endometriosis focus on either surgical removal of disease or hormonal suppression of lesion growth but this does not improve pain symptoms in all women (Taylor *et al.* 2017, Saraswat *et al.* 2018, Pokrzywinski *et al.* 2020). Furthermore, symptomatic relapse or further surgery occurs in 24–72% of women within 7 years of the initial surgery (reviewed in Guo & Martin 2019). We have previously shown that sham surgery in the peritoneal cavity of mice dramatically alters the composition of peritoneal macrophages by promoting increased replenishment by blood monocytes (Bain *et al.* 2020). Notably, this was detectable for up to 8 weeks post-surgery consistent with surgical intervention having a lasting effect on the peritoneal inflammatory environment in mice (Bain *et al.* 2020). In the context of the current results, we, therefore, speculate that surgical treatment itself could alter the peritoneal environment which raises the possibility that surgery could exacerbate pain symptoms in some patients. This may explain some of the contradictory findings assessing the impact of surgery on pain symptoms in women with superficial peritoneal endometriosis (Horne *et al.* 2019). The results of the current study suggest that assessing peritoneal macrophages could provide useful insights into the impact of surgical treatment as well as the inflammatory

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profile of patients with recurrent pain. Our new data also highlights the possibility that pain responses to non-surgical treatments may be influenced by peritoneal macrophage function. For example, GnRH agonists which reduce pelvic pain in women with endometriosis (Brown *et al.* 2010) can increase PMφ cytotoxicity (Braun *et al.* 1992) and reduce peritoneal fluid concentrations of the pain-correlated cytokine IL6 (Kuroda *et al.* 2010). GnRH modulators do not improve pain symptoms in all women (Taylor *et al.* 2017, Pokrzywinski *et al.* 2020) which could be accounted for by baseline differences in peritoneal macrophage phenotype or function. We, therefore, propose that profiling PMφ may have future utility for predicting response to surgical treatment and as a means for assessing the impact of medical treatments on inflammatory processes that drive pain symptoms.

Conclusions

The novel findings in this study provide new evidence for a link between the abundance of peritoneal macrophage subpopulations and the experience of pelvic pain symptoms in women. Collectively, our data suggest that the inflammatory profile of the peritoneal environment may be a better predictor of pain symptoms than the presence/volume of endometriosis lesions identified during laparoscopy. Reframing this perspective opens a path to future targeted therapies that aim to alter peritoneal macrophage function and reduce pelvic pain symptoms in women.

Supplementary materials

This is linked to the online version of the paper at https://doi.org/10.1530/RAF-20-0072.

Declaration of interest

A W H has received honoraria for consultancy for Ferring, Roche, Nordic Pharma, and Abbvie. B D L is now an employee of Bayer AG Pharma, Preclinical Research. Andrew Horne is a Co-Editor-in-Chief of Reproduction and Fertility. Andrew Horne was not involved in the review or editorial process for this paper, on which he is listed as an author.

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Author contribution statement

Experimental design; D A G, A W H, P T K S, experimental procedures; D A G, B D, F C, manuscript preparation; D A G, F C, A W H, P T K S.

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